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# THE IDENTIFICATION AND BIOSYNTHESIS OF TWO CYANOGENIC GLYCOSIDES IN *THALICTRUM AQUILEGIFOLIUM*

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**Abstract**—Three cyanogenic constituents have been detected in *Thalictrum aquilegifolium*. The structures of two of these have been confirmed as *p*-glucosyloxymandelonitrile and *p*-glucosyloxymandelonitrile β-glucoside. These two compounds have been shown to be biosynthetically derived from tyrosine.

## INTRODUCTION

DURING the last few years interest has revived in the cyanogenic glycosides found in plants. These compounds on treatment with dilute acids or suitable hydrolytic enzymes liberate HCN, one or more sugar molecules and an aldehyde or ketone. Biosynthetic studies have shown that the cyanogenic glycosides are derived from amino acids of related structure, e.g. dhurrin (*p*-hydroxymandelonitrile β-glucoside) from tyrosine[1] and linamarin (α-hydroxyisobutyronitrile β-glucoside) from valine.[2]

Early work by Itallie[3] had indicated that the leaves of *Thalictrum aquilegifolium* (Ranunculaceae) liberated HCN (about 0·05 per cent of fresh weight) on distillation after being ground and allowed to stand at 30° for some time. Stems liberated much smaller amounts of HCN while the roots liberated none at all. In addition to HCN, acetone was also detected in the distillate. Although there were no quantitative data on the relative amounts of HCN and acetone liberated it was postulated that the cyanogenetic principle was an acetone cyanhydrin, probably similar to linamarin. Such a compound might be expected to be derived from valine, as is linamarin. However, it has been shown[4] that tyrosine and not valine is incorporated into the cyanogens of *Thalictrum*. Because of these apparently conflicting pieces of evidence it was decided to re-investigate the cyanogenetic composition of *T. aquilegifolium*. This paper reports some studies on two of the cyanogenic glycosides found in this plant.

## RESULTS

### *Structure Elucidation*

The presence of three cyanogenic constituents in *Thalictrum aquilegifolium* was revealed by paper chromatography using *n*-butanol–ethanol–water followed by cyanide detection, elution and assay. The relative amounts of cyanide and $R_f$ values* are given in Table 1.

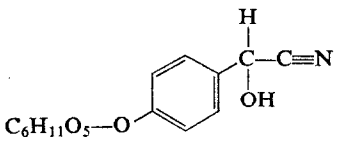
* $R_f$ values were obtained by cutting smaller sections from chromatograms for detection of cyanide or by rechromatography of purified material and detection by spraying the chromatogram with 0·1 M alkaline $KMnO_4$.

[1] J. KOUKOL, P. MILJANICH and E. E. CONN, *J. Biol. Chem.* **237**, 10 (1962).
[2] G. W. BUTLER and B. G. BUTLER, *Nature* **187**, 780 (1960).
[3] L. VAN ITALLIE, *Pharm. Weekblad.* **47**, 442 (1910).
[4] Y. P. ABROL, E. E. CONN and J. R. STOKER, unpublished results.

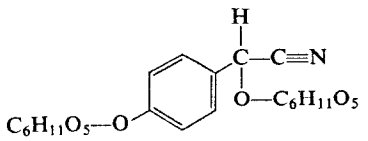
TABLE 1. CYANIDE CONTENT OF *T. aquilegifolium*

| $R_f$ value in butanol–ethanol–water (40:11:19) | Cyanide content ($\mu$moles/g fresh weight) |
|---|---|
| 0·06 | 7·70 |
| 0·35 | 0·61 |
| 0·72 | 0·43 |
| — | 8·3 from crude extract |

The major cyanogenic constituent was found to have an aromatic aglycone ($\lambda_{max}$ 280 nm) and biosynthetic studies have indicated that the compound is derived from L-tyrosine thus ruling out an acetone cyanhydrin type compound. Further work on the structure of this compound is in progress.




(I)




(II)

The two minor cyanogenic constituents (I and II) were further purified by paper chromatography in several solvent systems (for $R_f$s, see Table 2). Solutions of I and II were then hydrolysed by emulsin and the aqueous solution, after aeration (with $N_2$) to remove cyanide, was examined for ether-extractable aldehydes or ketones and for sugars. Both I and II gave the same aldehyde (detected by spraying with 0·1 % w/v 2,4-dinitrophenylhydrazine in 2 N HCl) of identical $R_f$ to *p*-hydroxybenzaldehyde in three solvent systems (using TLC and paper). The u.v. absorption curves of the aldehyde in ethanol (and with NaOH) were identical to those of *p*-hydroxybenzaldehyde. The aqueous solutions remaining after ether extraction gave glucose as the only sugar, identified by co-chromatography on paper and TLC. Quantitative determinations were now made of the HCN, *p*-hydroxybenzaldehyde and glucose liberated on enzymic hydrolysis, with the results as shown in Table 3.

TABLE 2. CHROMATOGRAPHIC MOBILITIES OF THE MINOR CYANOGENIC CONSTITUENTS

| Solvent | $R_f$ values of Compound I | $R_f$ values of Compound II |
|---|---|---|
| *n*-Butanol–ethanol–water (40:11:19) | 0·72 | 0·35 |
| *iso*-Propanol–water (7:3) | 0·72 | 0·55 |
| *n*-Butanol–acetic acid–water (12:3:5) | 0·82 | 0·85 |
| 5 % Acetic acid | 0·65 | 0·38 |

TABLE 3. HYDROLYSIS PRODUCTS OF TWO CYANOGENIC CONSTITUENTS OF *T. aquilegifolium*

| Compound | Hydrolysis products (μmoles) | | |
|---|---|---|---|
| | HCN | *p*-Hydroxybenzaldehyde | Glucose |
| Compound I | 2·26 | 1·97 | 2·1 |
| | 0·73 | — | 0·86 |
| Compound II | 0·22 | 0·20 | — |
| | 0·67 | 0·66 | 1·38 |
| | 1·51 | — | 3·80 |

Finally, the u.v. absorption spectra of the two cyanogenic constituents and of synthetic *p*-glucosyloxymandelonitrile were found to be similar ($\lambda_{max}$ in each case 270 nm).

*Biosynthetic Studies*

L-Tyrosine-1-$^{14}$C, DL-tyrosine-2-$^{14}$C, DL-tyrosine-3-$^{14}$C and L-tyrosine-U-$^{14}$C were administered to cut shoots of *T. aquilegifolium* and the two minor cyanogenic constituents isolated and purified by chromatography. The distribution in activity in the glycosides, aglycones and HCN was then measured (Tables 4 and 5).

TABLE 4. INCORPORATION OF RADIOACTIVITY INTO THE CYANIDE PORTION OF I AFTER ADMINISTRATION OF LABELLED TYROSINE

| Compound administered | Specific activity (μc/μmole) | Amount fed (μc) | HCN liberated (μmoles) | Activity in HCN (dpm/μmole) |
|---|---|---|---|---|
| L-Tyrosine-U-$^{14}$C* | 95·0 | 10·0 | 0·12 | $1·14 \times 10^4$ |
| L-Tyrosine-1-$^{14}$C | 26·6 | 1·0 | 8·5 | 0·0 |
| DL-Tyrosine-2-$^{14}$C | 1·36 | 1·5 | 5·8 | $6·2 \times 10^4$ |
| DL-Tyrosine-3-$^{14}$C | 6·85 | 2·0 | 8·2 | $0·025 \times 10^4$ |

* Activity incorporated into aglycone: $7·8 \times 10^4$ dpm/μmole, i.e. approximately seven times the activity of the cyanide portion.

TABLE 5. INCORPORATION OF RADIOACTIVITY INTO THE CYANIDE PORTION OF II AFTER ADMINISTRATION OF LABELLED TYROSINE

| Compound administration | Specific activity (μc/μmole) | Amount fed (μc) | HCN liberated (μmoles) | Activity in HCN (dpm/μmole) |
|---|---|---|---|---|
| L-Tyrosine-U-$^{14}$C* | 95·0 | 10·0 | 0·29 | $0·325 \times 10^4$ |
| L-Tyrosine-1-$^{14}$C | 26·6 | 1·0 | 8·5 | 0·0 |
| DL-Tyrosine-2-$^{14}$C | 1·36 | 1·5 | 5·8 | $1·55 \times 10^5$ |
| DL-Tyrosine-3-$^{14}$C | 6·85 | 2·0 | 8·2 | $0·026 \times 10^4$ |

* Activity incorporated into aglycone: $2·23 \times 10^4$ dmp/μmole, i.e. approximately seven times the activity of the cyanide portion.

## DISCUSSION

The two minor cyanogenic glycosides isolated from *Thalictrum aquilegifolium* each liberate *p*-hydroxybenzaldehyde, glucose and HCN on hydrolysis with almond emulsin. Compound I shows a ratio of 1·0:1·06:1·15 for *p*-hydroxybenzaldehyde to glucose to HCN. The u.v. absorption curve of the glycoside shows no bathochromic shift in the presence of alkali thereby confirming the absence of a free phenolic grouping. Consequently compound I must be *p*-glucosyloxymandelonitrile, confirmed by comparison with synthetic material. This compound has previously been found as a constituent of *Nandina domestica*.[5]

The chromatographic mobility of II indicates that it is more polar than I. The ratio for *p*-hydroxybenzaldehyde to glucose to HCN is 1·0:2·08:1·01. Once again u.v. evidence indicates the absence of a free phenolic group. Two structures are possible; the two glucose units could be in the form of a disaccharide on the phenolic group or, alternatively, both the phenolic and side-chain hydroxyl groups could be glucosylated. The former compound would be, like I, unstable in solution, slowly liberating HCN on storage. However, II is stable under such conditions and hence must be *p*-glucosyloxymandelonitrile β-glucoside, a compound not previously identified in plants.

The biosynthetic studies confirm that both I and II are derived from tyrosine. Only the α-carbon atom (from tyrosine-2-$^{14}$C) is appreciably incorporated into the nitrile group. Tyrosine-U-$^{14}$C results in the expected distribution of radioactivity between the *p*-hydroxybenzaldehyde and HCN of approximately 7:1 in both compounds. Consequently the biosynthesis of these two cyanogenic compounds from *T. aquilegifolium* seems to follow the same pattern as for the structurally related compound dhurrin[1] in *Sorghum vulgare* and of *p*-glucosyloxymandelonitrile itself in *Nandina domestica*.[5]

## EXPERIMENTAL

### *Plant Material*

*Thalictrum aquilegifolium* plants were grown from seed either in the greenhouse or outdoors.

### *Radioactive Compounds*

L-Tyrosine-1-$^{14}$C and DL-tyrosine-3-$^{14}$C were purchased from the New England Nuclear Corporation; DL-tyrosine-2-$^{14}$C from Volk Radiochemicals and L-tyrosine-U-$^{14}$C from the Radiochemical Centre at Amersham.

### *Administration of Labelled Compounds*

All radioactive compounds were administered in neutral aqueous solutions to cut shoots of the plants. The cut ends of shoots were trimmed under water before immersion in the tracer solution. Plants were given 16-hr illumination during the 24-hr metabolic period.

### *Analytical Methods*

Cyanide was determined by the cyanogen bromide method of Aldridge,[6] after liberation of HCN from cyanogenic compound by either of two methods. In the first method the material was heated in a boiling water bath for 15 min with 5 ml of 0·1 M NaOH. The solution was then cooled and aliquots used for the assay. This method proved useful in the rapid detection of cyanogenic compounds on sections cut from chromatograms. The second method was to liberate HCN from the cyanogenic glycoside by treatment with emulsin at pH 5·5 for 2 hr in a closed system. The HCN was then removed from the solution by moderate aeration with $CO_2$-free nitrogen and trapped in 10 ml of 0·1 M NaOH. Aliquots of the trapping solution were then used for the assay.

The aqueous solution from method two, now depleted of cyanide, was extracted with ether and the ether extract used in the spectral determination of *p*-hydroxybenzaldehyde. The ether extract was taken to dryness

[5] Y. P. ABROL, E. E. CONN and J. R. STOKER, *Phytochem.* **5**, 1021 (1966).
[6] W. N. ALDRIDGE, *Analyst* **69**, 262 (1944).

and the residue dissolved in ethanol (1 ml) and the u.v. spectrum obtained with and without the addition of one drop of 1·0 N NaOH.

The glucose present in the aqueous solution after extraction with ether was quantitatively measured by oxidation by glucose oxidase to glucuronic acid and $H_2O_2$.[7] The $H_2O_2$ in the presence of horse radish peroxidase oxidizes *o*-dianisidine to a red–brown compound which produces a purple colour with 5 N $H_2SO_4$. The optical density was measured at 525 nm. A modified Somogyi method[8] was also used to measure total sugars.

*Determination of Radioactivity*

Radioactivity measurements were made in Brays' solution[9] or in Toluene–Triton X-100 solution[10] using an I.D.L. liquid scintillation counter. *n*-Hexadecane-1-$^{14}$C was used as an internal standard.

*Isolation of Cyanogenic Compounds*

Shoots of *T. aquilegifolium* (76 g) were ground to a powder with liquid $N_2$. The powder was immediately transferred to boiling 80% v/v ethanol (400 ml) for 5 min. The suspension was centrifuged and the supernatant evaporated to dryness below 40°. The resultant residue was dissolved in 20% v/v ethanol (50 ml), stored at 4° for 24 hr, centrifuged and the supernatant evaporated to dryness. The residue was dissolved in 50% v/v ethanol (5 ml), an aliquot being taken for cyanide analysis, and the remainder for separation and purification of the cyanogenic compounds.

The ethanolic extract was streaked onto several sheets of Whatman No. 1 chromatography paper and the chromatograms developed with *n*-butanol–ethanol–water (40:11:19 v/v). The cyanogenic constituents were located on narrow strips cut from the chromatograms. Each of these strips was cut into ten equal sections and the cyanide was liberated by heating with NaOH solution as described above. The areas containing cyanide were then cut from the chromatograms and were each eluted by shaking with 50% ethanol. After bulking the corresponding fractions from each chromatogram the eluates were made to known volume and aliquots assayed for cyanide. Two of the cyanogenic constituents were then further purified by chromatography using *iso*-propanol–water (7:3 v/v), *n*-butanol–acetic acid–water (12:3:5 v/v) and 5% v/v acetic acid.

*Synthesis of* p-*Glucosyloxymandelonitrile*

Tetra-acetyl-*p*-glucosyloxybenzaldehyde was prepared according to the method of Robertson and Waters.[11] Deacetylation to *p*-glucosyloxybenzaldehyde was achieved by standing overnight at 5° with methanolic ammonia. Conversion to the cyanohydrin was then carried out with 10% v/v hydrogen cyanide solution essentially by the method of Fischer.[12] Overall yield 30%; m.p. 160–164°. Calculated for $C_{14}H_{17}O_7N$: C, 54·01; H, 5·46. Found: C, 53·40; H, 5·72 per cent.

*Acknowledgement*—The authors wish to express their appreciation to Professor Eric E. Conn, University of California at Davis, in whose laboratory this work was started. One of us (D. S.) would like to thank the Science Research Council for the award of a research studentship.

[7] J. D. Fleming and H. F. Pegler, *Analyst* **88**, 967 (1967).
[8] R. Nelson, *J. Biol. Chem.* **153**, 375 (1944).
[9] D. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
[10] M. S. Patterson and R. C. Greene, *Analyt. Chem.* **37**, 854 (1965).
[11] A. Robertson and R. B. Waters, *J. Chem. Soc.* 2739 (1930).
[12] E. Fischer, *Ber. Deut. Chem. Ges.* **34**, 630 (1901).